JC10 Rec'd PCT/PTO 19 JUN 2001

FORM PTO-1390 (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371

ATTORNEY'S DOCKET NUMBER: **24615-20145.00**

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/869088

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/NL99/00783	17/12/99	22/12/98

TITLE OF INVENTION

PROCESS FOR THE PREPARATION OF COMPOUNDS WITH ENHANCED OPTICAL PURITY

APPLICANT(S) FOR DO/EO/US

QUAEDFLIEG, Peter J.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ has been communicated by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)).
   - a. ☐ is attached hereto.
   - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ have been communicated by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information: *; return receipt postcard.

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

Express Mail Label No.: EL 719482167 US Date of Deposit: June 19, 2001

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

K. S. Huff

JC18 Rec'd PCT/PTO 19 JUN 2001

U.S. APPLICATION NO (if known, see 37 CFR 1.5) 09/869088	INTERNATIONAL APPLICATION NO PCT/NL99/00783	ATTORNEY'S DOCKET NUMBER. 24615-20145.00

21. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

	CALCULATIONS	PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,000.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..........$710.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provision of PCT Article 33(1)-(4) $690.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$*	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$*	
Total claims	19 - 20 =	*	x $18.00	$*	
Independent claims	2 - 3 =	*	x $80.00	$*	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$*	

TOTAL OF ABOVE CALCULATIONS =	$860.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.	$*	
SUBTOTAL =	$860.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$*	
TOTAL NATIONAL FEE =	$860.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00 per property** +	$40.00	
TOTAL FEES ENCLOSED =	$900.00	
	Amount to be refunded:	$*
	charged:	$*

a. ☒ A check in the amount of $900.00 to cover the above fees is enclosed.

b. ☐ Please charge my **Deposit Account No. 03-1952** in the amount of $* to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment to **Deposit Account No. 03-1952**. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Kate H. Murashige
Morrison & Foerster LLP
3811 Valley Centre Drive
Suite 500
San Diego, California 92130-2332

Reg. N. 39,183

SIGNATURE

for Kate H. Murashige
Registration No. (29,959)

09/869088

PATENT

Docket No. 246152014500

JC18 Rec'd PCT/PTO 19 JUN 2001

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

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K.S. Huff

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re: U.S. National Phase application of:

Peter J. Quaedflieg, *et al.*

Serial No.: Not yet assigned
Based on PCT Int'l App: PCT/NL99/00783

Int'l Filing Date: December 17, 1999

For: PROCESS FOR THE PREPARATION OF COMPOUNDS WITH ENHANCED OPTICAL PURITY

Examiner: Not yet assigned

Group Art Unit: Not yet assigned

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

This is a preliminary amendment prior to examination, please amend the claims as follows:

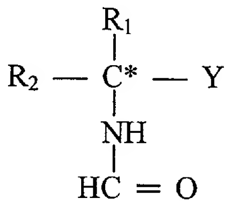
Enclosed is the following Exhibit A:

Exhibit A: Marked-up Version of Amendments to the Claims.

AMENDMENT

Please replace presently pending claims 1-11 with the following claims 1-11:

1. (Amended) A process for the preparation of a compound with enhanced optical purity which comprises contacting a mixture of the enantiomers of a chiral compound of formula 1



(1)

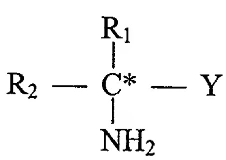
wherein:

R_1 represents an alkyl or an aryl group

R_2 represents H, an alkyl or an aryl group

Y represents an alkyl group, an aryl group, $(CH_2)_nCOOH$, $(CH_2)_n$-COOR, $(CH_2)_n$-CONRR′, CH_2OH, or C ≡N wherein R and R′ independently represent H, an alkyl or aryl group, and n represents 0 or 1, with an enzyme having peptide deformylase activity and with a bivalent metal ion as a cofactor, wherein the metal is a metal of the groups 5-11 of the periodic system, wherein one of the enantiomers is selectively deformylated.

2. (Amended) A process for the preparation of a compound with enhanced optical purity which comprises contacting a mixture of the enantiomers of a chiral compound of formula 2



(2)

wherein:

R_1 represents an alkyl or an aryl group

R_2 represents H, an alkyl or an aryl group

Y represents an alkyl group, an aryl group, $(CH_2)_nCOOH$, $(CH_2)_n$-COOR, $(CH_2)_n$-CONRR′, CH_2OH, or C ≡N wherein R and R′ represent H, an alkyl or aryl group, and n represents 0 or 1, with an enzyme having peptide deformylase activity, a bivalent metal ion as a

cofactor wherein the metal is of the groups 5-11 of the periodic system, and with a formylating agent, whereby one of the enantiomers is selectively converted in the corresponding N-formyl compound.

3. (Amended) The process of claim 2 wherein the formylating agent is formic acid, a formic acid amide or a formic acid ester.

4. (Amended) The process of any one of claim 1, wherein the peptide deformylase is of the class EC 3.5.2.27 or EC 3.5.1.31.

5. (Amended) The process of claim 1, wherein the peptide deformylase contains the sequences (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.

6. (Amended) The process of claim 1, wherein the peptide deformylase is obtainable from *Escherichia coli.*

7. (Amended) The process of claim 1, wherein the bivalent metal is Fe, Ni, Mn or Co.

8. (Amended) The process of claim 7, wherein the bivalent metal is Ni.

9. (Amended) The process of claim 1, which further comprises adding a stabilisation agent.

10. (Amended) The process of claim 9 wherein the stabilisation agent is catalase.

11. (Amended) The process of claim 9 wherein the bivalent metal is Fe.

Please add the following new claims:

12. (New) The process of any one of claim 2, wherein the peptide deformylase is of the class EC 3.5.2.27 or EC 3.5.1.31.

13. (New) The process of claim 2, wherein the peptide deformylase contains the sequences (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.

14. (New) The process of claim 2, wherein the peptide deformylase is obtainable from *Escherichia coli.*

15. (New) The process of claim 2, wherein the bivalent metal is Fe, Ni, Mn or Co.

16. (New) The process of claim 15, wherein the bivalent metal is Ni.

17. (New) The process of claim 2, which further comprises adding a stabilisation agent.

18. (New) The process of claim 17 wherein the stabilisation agent is catalase.

19. (New) The process of claim 18 wherein the bivalent metal is Fe.

REMARKS

The claims have been amended to eliminate multiple claim dependencies and to conform to U.S. practice. The changes to the claims are editorial and do not constitute new matter. Entry of the amendment is respectfully requested.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicants petition for any required relief including extensions of time and authorize the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket No. 246152014500. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: June 19, 2001

By: C J A A Reg No. 39,183
for Kate H. Murashige
Registration No. 29,959

Morrison & Foerster LLP
3811 Valley Centre Drive,
Suite 500
San Diego, California 92130-2332
Telephone: (858) 720-5112
Facsimile: (858) 720-5125

EXHIBIT A. - VERSION WITH MARKINGS TO SHOW CHANGES MADE

1. (Amended) [Process] <u>A process</u> for the preparation of a compound with enhanced optical purity [wherein] <u>which comprises contacting</u> a mixture of the enantiomers of a chiral compound of formula 1

$$\begin{array}{c} R_1 \\ | \\ R_2 - C^* - Y \\ | \\ NH \\ | \\ HC = O \end{array} \qquad (1)$$

wherein:

R_1 represents an alkyl or an aryl group

R_2 represents H, an alkyl or an aryl group

Y represents an alkyl group, an aryl group, $(CH_2)_nCOOH$, $(CH_2)_n$-COOR, $(CH_2)_n$-CONRR′, CH_2OH, or C ≡N wherein R and R′ independently represent H, an alkyl or aryl group, and n represents 0 or 1, [is brought into contact] with an enzyme having peptide deformylase activity <u>and</u> with a bivalent metal ion as a cofactor<u>,</u> wherein the metal is [chosen from] <u>a metal of</u> the groups 5-11 of the periodic system<u>, wherein one of the enantiomers is selectively deformylated</u>.

2. (Amended) [Process] <u>A process</u> for the preparation of a compound with enhanced optical purity [wherein] <u>which comprises contacting</u> a mixture of the enantiomers of a chiral compound of formula 2

$$\begin{array}{c} R_1 \\ | \\ R_2 - C^* - Y \\ | \\ NH_2 \end{array} \qquad (2)$$

wherein:

R_1 represents an alkyl or an aryl group

R_2 represents H, an alkyl or an aryl group

Y represents an alkyl group, an aryl group, $(CH_2)_nCOOH$, $(CH_2)_n$-COOR, $(CH_2)_n$-CONRR′, CH_2OH, or C ≡N wherein R and R′ represent H, an alkyl or aryl group, and n represents 0 or 1, [is subjected to a formylation reaction in the presence of an] <u>with an</u> enzyme

having peptide deformylase activity, [with] a bivalent metal ion as a cofactor wherein the metal is [chosen from] of the groups 5-11 of the periodic system, and with a formylating agent, whereby one of the enantiomers is selectively converted in the corresponding N-formyl compound.

3. (Amended) [Process] The process [according to] of claim 2 wherein the formylating agent is formic acid, a formic acid amide or a formic acid ester[is used as a formylating agent].

4. (Amended) [Process] The process [according to] of any one of [claims 1-3] claim 1, wherein the peptide deformylase is [chosen from] of the class EC 3.5.2.27 or EC 3.5.1.31.

5. (Amended) [Process] The process [according to any] of [claims 1-4] claim 1, wherein the peptide deformylase contains the sequences [of] (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.

6. (Amended) [Process] The process [according to any] of [claims 1-5] claim 1, wherein the peptide deformylase is obtainable from *Escherichia coli.*

7. (Amended) [Process] The process [according to any] of [claims 1-6] claim 1, wherein the bivalent metal is [chosen from the group of] Fe, Ni, Mn [and] or Co.

8. (Amended) [Process] The process [according to] of claim 7, wherein the bivalent metal is Ni.

9. (Amended) [Process] The process [according to any] of [claims 1-8] claim 1, [wherein in addition] which further comprises adding a stabilisation agent[is added].

10. (Amended) [Process] The process [according to] of claim 9 wherein the stabilisation agent is catalase.

11. (Amended) [Process] The process [according to] of claim 9 [or 10] wherein the bivalent metal is Fe.

PROCESS FOR THE PREPARATION OF COMPOUNDS WITH ENHANCED OPTICAL PURITY

The invention relates to a process for the preparation of a compound with enhanced optical purity wherein a mixture of the enantiomers of a chiral compound of formula 1:

```
         R1
         |
  R2 - C* - Y          (1)
         |
         NH
         |
       HC = O
```

wherein:
R_1 represents an optionally substituted alkyl or an optionally substituted aryl group
R_2 represents H, an optionally substituted alkyl or an optionally substituted arylgroup
Y represents an alkyl group, an aryl group, $(CH_2)_nCOOH$, $(CH_2)_n$-COOR, $(CH_2)_n$-CONRR', CH_2OH, or C≡N wherein R and R' independently represent H, an alkyl or aryl group, and n represents 0 or 1, is brought into contact with an enzyme having peptide deformylase activity with a bivalent metal ion as a cofactor wherein the metal is chosen from the groups 5-11 of the periodic system.

Enzymes having peptide deformylase activity are known in the literature e.g. from P.T. Ravi Rajagopalan et al., Biochemistry 1997, 36, 13910-13918 wherein the use of peptide deformylase is described for

Enclosure 1.1 9873WO

AMENDED PAGE 2

the deformylation of several peptides with N-formylmethionine at the N-terminus and of N-formylmethionine. Although the known peptide deformylases showed reasonable deformylase activity when peptides where used as a substrate, they showed no or little activity with respect to N-formylmethionine.

Applicant surprisingly found that the peptide deformylases having a bivalent metal ion as a cofactor according to the invention, did not only show a considerable activity towards the substrates according to formula 1, but also appeared to be enantioselective. *US-A-4,745,067 discloses L-aminoacylases which exhibit enantioselective activity towards N-acyl-L-amino acids. However, the activity of these enzymes towards N-formyl-L-methionine is low.*

The alkyl groups in R_1, R_2, R, R^1 en Y may be cyclic or linear or branched chains. The alkyl, aryl and the methylene groups may be substituted. Suitable substituents are for instance, hydroxy, alkyl, alkoxy, e.g. methoxy, mercapto, alkylmercapto, amino, guanyl, carboxamide, halogen, e.g. chloro, aryl, e.g. phenyl and hydroxyphenyl, imidazolyl or indonyl.

Substrates according to formula 1 that can be used in the process of the invention are for instance amino acids, for instance α- or β-amino acids with 1-20 C-atoms, in particular α-H-α-amino acids, α-methyl-α-amino acids, β-amino acids; esters of said amino acids wherein the ester group is for instance an alkyl group having 1-10 C-atoms; amides of said amino acids, wherein optionally the amide is N-substituted with 1 or 2, preferably 1, substituent chosen from alkyl or aryl, having 1-10 C-atoms; nitriles corresponding to said α-amino acids; amino alcohols corresponding to said α-amino acids; or amines for instance (optionally substituted) aromatic or aliphatic

amines. Suitable substituents are for instance (optionally substituted) alkyl groups, for instance with 1-10 C-atoms.

In another embodiment of the present invention a mixture of the enantiomers of a (non protected) amino compound is subjected to a formylation in the presence of a peptide deformylase having a bivalent metal ion as a cofactor, wherein the metal is chosen from the groups 5-11 of the periodic system and a formylating agent, whereby one of the enantiomers is selectively converted into the corresponding N-formyl amino compound.

Suitable formylating agents are for instance formic acid in case a thermodynamically controlled formylation can be performed, or formic acid esters or amides when the formylation is kinetically controlled. In a thermodynamically controlled formylation the equilibrium is shifted towards the side of formyl derivative, preferably by precipitation of the formyl derivative.

Peptide deformylases are in general enzymes having formyl methionine peptide deformylase activity. The peptide deformylases to be used according to the invention have a more than 10 times, preferably more than 100 times, in particular more than 1000 times, higher activity towards the formyl protected compounds according to formula 1 compared to the corresponding acetyl protected compounds. Activity here is defined as the catalytic efficiency (also called: specificity constant) K_{cat}/K_m expressed in M^{-1} sec^{-1}; wherein K_m (expressed in mM) represents the Michaelis constant (this is the substrate concentration at which the reaction rate is 50% of the maximum reaction rate observed) and K_{cat} (expressed in min^{-1}) represents the

turnover number. It should be noticed that in the literature also other names are being used instead of the name Peptide deformylases; in particular the following names may be mentioned here: formylmethionine deformylase, N- formylmethionyl aminoacyl-tRNA deformylase, N-formyl-L-methionine amidohydrolase N-formylmethionyl-aminoacyl-tRNA amidohydrolase.

Suitable peptide deformylases to be used in the process according to the invention are peptide deformylases classified as EC 3.5.1.27. Preferably, the enzyme is an enzyme having the activity as described for EC 3.5.1.27 because excellent results are being achieved in the deformylation with such enzymes. It should be noticed, that until recently it was believed that the enzyme coded as EC 3.5.1.31 is catalyzing a different reaction. In the meantime however it has been shown that the enzymes known as EC 3.5.1.27 and EC 3.5.1.31 are coded for by exactly the same gene and have the same activity. Therefore, as used herein, the term EC 3.5.1.27 is encompassing not only EC 3.5.1.31, but likewise all other enzymes having the same activity as described for EC 3.5.1.27.

Although the family of PDF's is composed of proteins with a relatively low level of sequence identity, the 3D structures of the members of this family appear closely related one to each other with, in particular, the building of a common fold around the bivalent metal ion and three signature sequences. As is described (for PDF's indicated as PDF) by Wagner et al., J. Biol. Chem., **273**, 11413-6 (1998), for many of these enzymes characteristically three short amino acid stretches are present as strictly conserved motifs, namely in that the enzymes contain the sequences (i)

HEXXH, (ii) EGCLS and (iii) GXGXAAXQ. In these sequences X represents any natural amino acid, and standard one letter codes for amino acids are used: A = alanine, C = cysteine, E = glutamic acid, G = glycine, H = histidine, L = leucine, S = serine and Q = glutamine.

Peptide deformylases are obtainable for instance from eubacteria for example *Escherichia coli, Bacillus subtilis, Clostridium acetobutylicum Clostridium beyerinckii, Haemophilus influenzae Thermotoga maritima, Thermus aquaticus, Thermus thermophilus, Calothrix PCC 7601, Bacillus stearothermophilus* or *Lactococcus lactis*. Preferably an enzyme of *Escherichia Coli* is used.

The peptide deformylases according to the invention require a bivalent metal ion whereby the metal is chosen from the groups 5-11 of the periodic system (New IUPAC version; see Handbook of Chemistry and Physics 70th edition, CRC Press, 1989-1990, inner page of cover), as a cofactor. Preferably the metal is chosen from the group of V, Cr, Fe, Ni, Mn, Co, Cu, Pd and Pt, in particular from the group of Fe, Ni, Mn and Co.

Preferably the amount of the bivalent metal ions should be about equivalent to the number of moles of enzyme. Suitably the molar ratio between these bivalent metal ions and the number of PDF molecules is in the range of 0.6 to 1.4, preferably of 0.8 to 1.2, and most preferred the amount of bivalent metal ions is equimolar to the enzyme.

Exchange of the bivalent metal ions in the

PDF's in order to obtain PDF enzymes with a co-factor as necessary for the present invention can be done by the various methods as described in Groche *et al.*, Biochem. Biophys. Res. Comm., **246**, 342-346, (1998). These methods include simple metal displacement by incubation of the native enzyme in an excess of the desired bivalent metal ion, if necessary preceeded by the preparation of the apoenzyme via treatment of the native enzyme with a metal chelation compound. Furthermore, the desired bivalent metal ion can already be introduced in (at least part of the enzyme molecules) by using a bacterial growth medium with an enhanced ratio of the desired bivalent metal ion over Fe^{2+}.

In addition measures may be taken in order to enhance the stability of the enzyme, for instance the addition of stabilisation agents, for instance catalase, tris-(2-carboxyethyl)phosphine, glucose oxidase, or combinations thereof; or enlarging the concentration of the PDF, for instance to a PDF concentration of at least 0.1 mg of PDF per ml, more preferably of least 1.0 mg/ml. The upper limit of the concentration of PDF is not critical if practical concentrations are being used. The use of stabilisation measures is especially preferred when an easily oxidisable metal ion, e.g. Fe^{++} is present as a cofactor or an easily oxidisable substrate. If not, for instance in case Ni^{++} is present as a cofactor the addition of a stabilisation agent appeared to be superfluous, as the enzyme turned out to be very stable even without

stabilisation agent.

The enzymes applied in the process according to the invention may be purified enzymes, a crude enzyme solution, microbial cells exhibiting the required activity, a homogenate of cells or permeabilized cells. If required, the enzyme may be applied in an immobilized state or in a chemically modified form to ensure a good stability, reactivity. and enantioselectivity of the enzymes under the conditions utilized.

Alternatively, genetically engineered mutants of PDF's may be used which have for instance enhanced activity or enantioselectivity in the (de)formylation reaction. These mutants can be generated by a number of different approaches; for instance, by site-directed mutagenesis, site-specific random mutagenesis, regio-specific random mutagenesis, and completely random mutagenesis; the latter form of mutagenesis is better known as directed evolution. General applicable methods to perform these different protein engineering approaches are well known to the skilled man. If a random approach will be applied, the mutagenesis cycle will need to be followed by selection of resistent and active mutant(s), thereby leading to the identification of suitable mutants. To obtain PDF mutants also a combination of different protein engineering approaches and/or several rounds of random mutagenesis may be used.

The reaction conditions for the enzymatic deformylation according to the invention are not very critical and may depend on the substrate used. Any

suitable solvent system which is inert towards the PDF may be applied; such solvents include aqueous systems (solutions or slurries) or aqueous systems also containing a water-miscible organic solvent which is inert under the reaction conditions. Aqueous systems, however, are preferred. Also the concentration of the *N*-formyl compound is not critical, and may be for instance in the range of about 0.1 to 1000 mM. It is not necessary that all of the *N*-formyl compound is dissolved; part of it may be present as a slurry. The concentration of the PDF likewise is not very critical, and usually will be at 0.001 to 100 % by weight of the formyl compound, e.g. at about 0.2 mM of PDF. The pH for the reaction preferably is chosen in the range of 4.0 to 11.0, more preferably of 5.0 to 10.0. The temperature is not very critical, and suitably will be in the range of 10 to 50^{o}C, e.g. at about 37^{o}C, but for thermostable PDF enzymes higher temperatures may be applied.

In those cases wherein the absolute configuration of the (de)formylated enantiomer was determined, it appeared that the S-enantiomer was (de)formylated more rapidly than the R-enantiomer. The optical purity is given by the enantiomeric excess (ee), the enantioselectivity of the enzyme is represented by E, and calculated as k_f/k_s wherein k_f is defined as the rate of (de)formylation of the most rapidly (de)formylated enantiomer and k_s is defined as the rate of (de)formylation of the least rapidly (de)formylated enantiomer.

Optionally a salt promoting hydrophobic interactions is added to the reaction mixture, for instance a sulphate, phosphate, sulphite or acetate of ammonium, Rb, K, Na, Cs or Li. Most preferably ammonium sulphate or lithium sulphate is used.

The invention will further be elucidated by the following examples, without being limited thereto.

Abbreviations:

TB medium:	12 g/l of Bacto-Tryptone, Difco; 24 g/l of yeast extract, Difco; 4 g/l of glycerole; 2.3 g/l of KH_2PO_4; 12.5 g/l of K_2HPO_4);
Hepes:	*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphuric acid;
AEBSF:	2-aminoethyl-p-benzene sulphonyl fluoride;
TCEP:	tris-(2-carboxyethyl)-phosphine.
MOPS:	3-(N-morpholino)propane sulphonic acid
MES:	2-(N-morpholino)ethane sulphonic acid

Examples 1-15, Comparative experiments A and B

Isolation of PDF(Fe)

For a detailed discussion of the methods used reference is made to Groche et al., BBRC **246,** 342-346 (1998).

PDF(Fe) was isolated from overproducing *E.coli* cells grown at 30°C in 1.6 l TB medium for 14-16 h. About 13 g (wet weight) cell paste were suspended in 26 ml buffer (20 mM Hepes/KOH, 100 mM KF, pH 7.7

supplemented with 10 μg/ml catalase from bovine liver (Boehringer Mannheim) and 1 mM AEBSF, disintegrated by sonication (Branson B12, 20 min) at 0°C and centrifuged at 200.000 g for 1 h. The clear supernatant (1.3 g of protein; according to biurete reaction) was mixed with 1.3 ml 10%(w/v) Polymin G-35 (BASF) adjusted to pH 7.7 and centrifuged at 40.000 g for 10 min. The supernatant was applied to a 20 ml Met-Lys-Sepharose column that had been equilibrated with 20 mM Hepes/KOH, 100 mM KF, 0.2 mM TCEP, pH 7.7. After washing with 120 ml of 20 mM Hepes/KOH, 100 mM KF, 0.2 mM TCEP, pH 7.7, PDF(Fe) was eluted with 150 ml 20 mM Hepes/KOH, 100 mM KCl, 0.2 mM TCEP, pH 7.7. The protein containing fractions were concentrated by ultrafiltration using an Amicon PM10 membrane (yield: 140 mg protein, 1400 U/mg; determined according to Groche et al.). After adjustment of the TCEP concentration to 1 mM and protein concentration to 40 mg/ml, the PDF(Fe) stock solution (40 mg/ml = 2 mM) was stored frozen at -60°C.

After thawing, the PDF(Fe) stock solution could be used directly in the deformylation experiments described below. If however solutions with lower PDF(Fe) concentrations were required for these deformylation experiments, the PDF stock solution was diluted further in 20 mM Hepes/KOH, pH 7.7, 100 mM KCl, 1 mg/ml bovine serum albumin, 10 μg/ml catalase solution.

<u>HPLC-analysis</u>

In all cases HPLC conditions had to be developed in which the two deformylated isomers were

separated from each other and from the formylated isomers. To this end two different techniques were applied that is method 1 and method 2, as described below.

From the quantities of deformylated isomers in the samples after various reaction times, both the initial deformylation rate constant (k_f and k_s in $M^{-1}s^{-1}$) could be calculated for both enantiomers, as well as the respective ee values. The enantioselectivity of the enzyme (E value) was calculated by taking the ratio of k_f/k_s and is given for all Examples in table 1, as well as the maximum ee value (ee_{max}) observed during the experiments.

Method 1 (without derivatization)

A Crownpak CR(+) column (4x150 mm) was used. Samples (5 µl) withdrawn from the deformylation mixture were mixed with 95 µl aqueous $HClO_4$ (10 mM) to inactivate PDF(Fe). Following a brief centrifugation, 20 µl of the supernatant were applied to the Crownpak CR(+) column. For specific chromatographic conditions and retention times see table 2.

Method 2 (Precolumn derivatization with o-phthaldialdehyde (OPA) and N-acetyl-L-cysteine (NAC).

Samples (25 µl) withdrawn from the deformylation mixture were mixed with 25 µl aqueous $HClO_4$ (100 mM) to inactivate PDF(Fe) . Following a brief centrifugation, 40 µl of the supernatant were added to 80 µl 1 M aqueous H_3BO_3/NaOH pH 9.4, subsequently 20 µl OPA

reagent (consisting of OPA in H_2O/CH_3OH 1:1 v/v with a concentration as indicated in table 3) and 20 µl NAC reagent (consisting of NAC in H_2O/CH_3OH 1:1 v/v with a concentration as indicated in table 3) was added. After the time indicated in table 3 derivatization was terminated by addition of 80 µl (250 mM) aqueous H_3PO_4, and 20 µl of the solution were instantaneously applied to a Nucleosil 120-5 C_{18} (250x4 mm) column. Temperature was always ambient and detection was spectrophotometric using a wavelength of 257 nm and/or 340 nm. The used eluent was a mixture of aqueous 0.05 M H_3PO_4 brought at pH 7.0 with 1 M NaOH, and a percentage of acetonitrile as indicated in table 3.
For derivatization of valine aminonitrile borate buffer was adjusted to pH 11 and addition of NAC reagent was done 10 min after OPA reagent had been added. Concentration of H_3PO_4 used for termination was 500 mM. Derivatization and separation conditions as well as the observed retention times for the deformylated compounds analyzed are compiled in table 3.

Examples 1-12 and comparative experiments A and B were executed according to the procedures A, B, or C as given below as indicated in table 1. The results of the examples and comparative experiments are summarized in table 1 and the corresponding HPLC conditions in tables 2 and 3.

Method A

Deformylation in the presence of Li_2SO_4 at pH 7.2

Deformylation reactions were performed in 1.5 ml Eppendorf reaction test tubes. The reaction mixture with a total volume of 200 µl contained 100 mM aqueous MOPS/NaOH, 2 M Li_2SO_4 buffer pH 7.2, and the concentration of formylated compound as indicated in table 1. After thermal equilibration to 37°C the deformylation reaction was started by the addition of the concentration of PDF as indicated in table 1. At various reaction times samples of the reaction mixture were withdrawn in which the reaction was stopped by addition of $HClO_4$.

Method B

Deformylation in the absence of Li_2SO_4 at pH 7.2

Reactions were performed as described in Method A with the exception that 100 mM aqueous MOPS/NaOH, 250 mM NaCl, 0.1 mg/ml catalase buffer pH 7.2 was used in stead of 100 mM aqueous MOPS/NaOH, 2 M Li_2SO_4 buffer pH 7.2.

Method C

Deformylation in the absence of Li_2SO_4 at pH 6.2

Reactions were performed as described in Method A with the exception that 100 mM aqueous MES/NaOH buffer pH 6.2 was used in stead of 100 mM MOPS/NaOH, 2 M Li_2SO_4 buffer pH 7.2.

Table 1
Results of deformylation experiments

Ex.	Compound	Type of compound	Method	[Compound] (mM)	[PDF] (μM)	k_s ($M^{-1}s^{-1}$)	k_f ($M^{-1}s^{-1}$)	E	ee_{max} (%)
1	*N*-formyl-phenylglycine	α-H-amino acid	B	10	200	0.0047	10,6	2255	99,6
2	*N*-formyl-3-amino-3-phenyl-propionic acid	β-H-amino acid	B	10	10	<0.004	7,1	>1775	100
3	*N*-formyl-phenylglycine amide	α-H-amino acid amide	B	10	5.2	0.09	227	2522	99,7
4	*N*-formyl-*tert*-leucine amide	α-H-amino acid amide	A	4.8	200	0.0005	0,15	300	100
5	*N*-formyl-α-methyl-phenylglycine amide	α-alkyl-amino acid amide	A	10	200	0.0005	0,045	90	100
6	*N*-formyl-phenylglycinol	β-amino alcohol	B	10	200	0.029	0,69	23,8	90,5
7	*N*-formyl-phenylglycinol	β-amino alcohol	A	10	200	0.34	6,3	18,5	93,3
8	*N*-formyl-alaninol	β-amino alcohol	A	10	200	0.018	0,22	12	85,6
9	*N*-formyl-phenylalanine aminonitrile	α-aminonitrile	C	7.5	20	1	880	880	98,8
10	*N*-formyl-valine aminonitrile	α-aminonitrile	A	10	50	0.62	29,7	47,9	95,5
11	*N*-formyl-*m*-methoxy-phenylalanine aminonitrile	α-aminonitrile	B	7.2	2.5	2	1370	685	99,0
12	*N*-formyl-1-(1-naphthyl)-ethylamine	amine	A	0.42	200	0.03	0,45	15	90
A	*N*-acetyl-phenylglycine amide	α-H-amino acid amide	A	10	10	<0.001	<0,001	-	-
B	*N*-formyl-proline	α-H-imino acid	A	10	200	<0.004	<0,004	-	-

Table 2:
Analytical conditions and retention times analyzed according to method 1

Ex.	Compound	Eluent	flow rate (ml/min)	T (°C)	Detection (nm)	retention time (min)		
						Amine	Amine	formyl-compound
1	*N*-formyl-phenylglycine	10 mM aq. $HClO_4$	1.0	40	210	2.1(D)	3.8(L)	9.6
2	*N*-formyl-3-amino-3-phenyl-propionic acid	85% 100 mM aq. $HClO_4$/15% CH_3OH	0.7	5	210	23.7	26.7	11.4
3	*N*-formyl-phenylglycine amide	10 mM aq. $HClO_4$	0.8	22	210	3.2(D)	12.6(L)	6.3
6/7	*N*-formyl-phenylglycinol	95% 10 mM aq. $HClO_4$/5% CH_3OH	0.8	5	210	4.8(L)	5.7(D)	10.0
9	*N*-formyl-phenylalanine aminonitrile	90% 10mM aq. $HClO_4$/10% CH_3OH	0.8	5	210	11.8	15.1	28.6
11	*N*-formyl-*m*-methoxy-phenylalanine aminontrile	90% 10mM aq. $HClO_4$/10% CH_3OH	0.8	5	210	23.8	30.7	52.0
12	*N*-formyl-1-(1-naphthyl)-ethylamine	85% 10 mM aq. $HClO_4$/15% CH_3OH	1.0	40	210	26.5(S)	31.2(R)	73.5
A	*N*-acetylphenylglycine amide	10 mM aq. $HClO_4$	0.8	22	210	3.2 (D)	12.6 (L)	6.9
B	*N*-formyl-proline	100 mM aq. $HClO_4$	0.4	5	200	3.8	3.8	5.7

Table 3:
Analytical conditions and retention times analyzed according to method 2

Ex.	**Compound**	**OPA (mg/ml)**	**NAC (mg/ml)**	**Time (min.**	**% CH_3CN**	**Retention Time (min.)**	**Amine Amineformyl**
2	*N*-formyl-3-amino-3-phenyl propionic acid	4	4	30	15	19.7	23.3
4	*N*-formyl-*tert*-leucine amide	8	8	10	22.5	14.9 (D)	17.4 (L)
5	*N*-formyl-α-methyl-phenylglycine amide	16	16	30	20	24.4	26.3
8	*N*-formyl-alaninol	4	4	5	15	16.9(L)	18.8(D)
10	*N*-formyl-valine aminonitrile	16	4	5	20	8.6 (L)	10.2 (D)

C L A I M S

1. Process for the preparation of a compound with enhanced optical purity wherein a mixture of the enantiomers of a chiral compound of formula 1

$$\begin{array}{c}
\quad\quad R_1 \\
\quad\quad | \\
R_2 \; - \; C^* \; - \; Y \\
\quad\quad | \\
\quad\quad NH \\
\quad\quad | \\
\quad HC = O
\end{array} \qquad\qquad (1)$$

te vaag daarom niet nieuw.

wherein:
R_1 represents an alkyl or an aryl group
R_2 represents H, an alkyl or an aryl group
Y represents an alkyl group, an aryl group, $(CH_2)_nCOOH$, $(CH_2)_n$-COOR, $(CH_2)_n$-CONRR', CH_2OH, or C≡N wherein R and R' independently represent H, an alkyl or aryl group, and n represents 0 or 1, is brought into contact with an enzyme having peptide deformylase activity with a bivalent metal ion as a cofactor wherein the metal is chosen from the groups 5-11 of the periodic system. → hoe dan acetyl

2. Process for the preparation of a compound with enhanced optical purity wherein a mixture of the enantiomers of a chiral compound of formula 2

$$\begin{array}{c}
\quad\quad R_1 \\
\quad\quad | \\
R_2 \; - \; C^* \; - \; Y \\
\quad\quad | \\
\quad\quad NH_2
\end{array} \qquad\qquad (2)$$

wherein:
R_1 represents an alkyl or an aryl group

R_2 represents H, an alkyl or an arylgroup Y represents an alkyl group, an aryl group, $(CH_2)_nCOOH$, $(CH_2)_n$-COOR, $(CH_2)_n$-CONRR', CH_2OH, or C≡N wherein R and R' represent H, an alkyl or aryl group, and n represents 0 or 1, is subjected to a formylation reaction in the presence of an enzyme having peptide deformylase activity with a bivalent metal ion as a cofactor wherein the metal is chosen from the groups 5-11 of the periodic system, and a formylating agent, whereby one of the enantiomers is selectively converted in the corresponding N-formyl compound

3. Process according to claim 2 wherein formic acid, a formic acid amide or a formic acid ester is used as a formylating agent.

4. Process according to any one of claims 1-3, wherein the peptide deformylase is chosen from the class EC 3.5.2.27 or EC 3.5.1.31.

5. Process according to any of claims 1-4, wherein the peptide deformylase contains the sequences of (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.

6. Process according to any of claims 1-5, wherein the peptide deformylase is obtainable from *Escherichia coli*.

7. Process according to any of claims 1-6, wherein the bivalent metal is chosen from the group of Fe, Ni, Mn and Co.

8. Process according to claim 7, wherein the bivalent metal is Ni.

9. Process according to any of claims 1-8, wherein in addition a stabilisation agent is added.

10. Process according to claim 9 wherein the stabilisation agent is catalase.

11. Process according to claim 9 or 10 wherein the

bivalent metal is Fe.

A B S T R A C T

Process for the preparation of a compound with enhanced optical purity wherein a mixture of the enantiomers of a chiral compound of formula 1

$$\begin{array}{c} R_1 \\ | \\ R_2 - C^* - Y \\ | \\ NH \\ | \\ HC = O \end{array} \qquad (1)$$

wherein:

R_1 represents an alkyl or an aryl group

R_2 represents H, an alkyl or an aryl group

Y represents an alkyl group, an aryl group, $(CH_2)_nCOOH$, $(CH_2)_n$-COOR, $(CH_2)_n$-CONRR', CH_2OH, or C≡N wherein R and R' independently represent H, an alkyl or aryl group, and n represents 0 or 1, is brought into contact with an enzyme having peptide deformylase activity with a bivalent metal ion as a cofactor wherein the metal is chosen from the groups 5-11 of the periodic system, or for the preparation of a formylated compound with enhanced optical purity from a mixture of the enantiomers of the corresponding not formylated chiral compound in the presence of a formylation agent.

Preferably the peptide deformylase is chosen from the class EC 3.5.2.27 or EC 3.5.1.31, and contains the sequences of (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ. The bivalent metal may be chosen from the group of Fe, Ni, Mn and Co, preferably Ni or Fe.

DECLARATION FOR *UTILITY/DESIGN] PATENT APPLICATION

AS A BELOW-NAMED INVENTOR, (I~) HEREBY DECLARE THAT:

(My) residence, post office address, and citizenship are as stated below next to (my) name(s~).

(I~) believe (i~) (am) the original, first and *[sole/joint] inventor(s~) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PROCESS FOR THE PREPARATION OF COMPOUNDS WITH ENHANCED OPTICAL PURITY .

the specification of which is attached hereto unless the following box is checked:

☒ was filed on 17 December 1999 as PCT International Application No. PCT/NL99/00783 and was amended on 16 October 2000.

(I~) hereby state that I have reviewed and understand the contents of the above-mentioned specification, including the claims, as amended by any amendment referred to above.

(I~) acknowledge the duty to disclose information which is material to the patentability as defined in 37 C.F.R. § 1.56.

(I~) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

Application No.	Country	Date of Filing (day/month/year)	Priority Claimed?
98204371.3	Europe	22 December 1998	☒Yes ☐No

(I~) hereby claim benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:/

Application Serial No.	Filing Date
*	

(I~) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, (i~) acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status
PCT/NL99/00783	17 December 1999	☐Patented ☒Pending ☐Abandoned

I hereby appoint the following attorneys and agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Randolph Ted Apple (Reg No. 36,429)
Sanjay S. Bagade (Reg No. 42,280)
Erwin J. Basinski (Reg No. 34,773)
Richard R. Batt (Reg No. 43,485)
Vincent J. Belusko (Reg No. 30,820)
Kimberly A. Bolin (Reg No. ~~44,546~~)
Tyler S. Brown (Reg No. 36,465)
A. Randall Camacho (Reg No. ~~46,595~~)
Robert K. Cerpa (Reg No. ~~39,933~~)
Alex Chartove (Reg No. 31,942)
Thomas E. Ciotti (Reg No. 21,013)
Matthew M. D'Amore (Reg No. 42,457)
Peter Davis (Reg No. 36,119)
Carolyn A. Favorito (Reg No. 39,183)
Hector Gallegos (Reg No. 40,614)
Debra J. Glaister (Reg No. 33,888)
Johney U. Han (Reg No. ~~45,565~~)
Alan S. Hodes (Reg No. ~~38,185~~)
Peter Hsieh (Reg No. ~~44,780~~)
Madeline I. Johnston (Reg No. 36,174)
Parisa Jorjani (Reg No. 46,813)
Richard C. Kim (Reg No. ~~40,046~~)
Kawai Lau (Reg No. 44,461)
Michael J. Mauriel (Reg No. ~~44,226~~)
Philip A. Morin (Reg No. P-45,926)
Martin M. Noonen (Reg No. 44,264)
Paul J. Riley (Reg No. 38,596)
Terri Shieh-Newton (Reg No. 47,081)
Kevin R. Spivak (Reg No. 43,148)
Michael R. Ward (Reg No. 38,651)
Todd W. Wight (Reg No. ~~45,218~~)
David T. Yang (Reg No. ~~44,415~~)
George C. Yu (Reg No. 44,418)
Laurie A. Axford (Reg No. 35,053)
Joseph Barrera (Reg No. ~~44,522~~)
Shantanu Basu (Reg No. ~~43,318~~)
Frank P. Becking (Reg No. 42,309)
Jonathan Bockman (Reg No. ~~45,640~~)
Barry E. Bretschneider (Reg No. ~~28,055~~)
Nicholas Buffinger (Reg No. ~~39,124~~)
Mark R. Carter (Reg No. 39,131)
Peng Chen (Reg No. ~~43,543~~)
Thomas Chuang (Reg No. 44,616)
Cara M. Coburn (Reg No. 46,631)
Raj S. Davé (Reg No. ~~42,465~~)
Stephen C. Durant (Reg No. 31,506)
David L. Fehrman (Reg No. 28,600)
Thomas George (Reg No. 45,740)
Kenneth R. Glick (Reg No. ~~28,612~~)
Douglas G. Hodder (Reg No. ~~41,840~~)
Charles D. Holland (Reg No. 35,196)
Wayne Jaeschke, Jr. (Reg No. ~~38,503~~)
Richard D. Jordan (Reg No. 33,519)
Ararat Kapouytian (Reg No. 40,044)
Cameron A. King (Reg No. 41,897)
Rimas T. Lukas (Reg No. ~~46,451~~)
Gladys H. Monroy (Reg No. 32,430)
Kate H. Murashige (Reg No. 29,959)
Catherine M. Polizzi (Reg No. ~~40,130~~)
Debra A. Shetka (Reg No. ~~33,309~~)
Rebecca Shortle (Reg No. 47,083)
Stanley H. Thompson (Reg No. 45,160)
E. Thomas Wheelock (Reg No. 28,825)
Frank Wu (Reg No. 41,386)
Peter J. Yim (Reg No. ~~44,417~~)
Karen R. Zachow (Reg No. 46,332)

and:

Please direct all communications to:

Morrison & Foerster LLP
3811 Valley Centre Drive
Suite 500
San Diego, California 92130-2332

Please direct all telephone calls to Morrison & Foerster at (858) 720-5100.

(I~) hereby declare that all statements made herein of (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

May 8th 2001

Date

100

Name: QUAEDFLIEG, ~~Peter, Jan, Leonard,~~ Mario
Residence: The Netherlands
Citizenship: Dutch
Post Office Address: Kochstraat 6, 6164 HB Geleen

May 9th, 2001

Date

Theo Sonke

200

Name. SONKE, Theodorus
Residence: The Netherlands NLX
Citizenship: Dutch
Post Office Address: Caeciliastraat 15, 6143 BK Guttecoven

May 25th, 2001

Date

Volker Wagner

300

Name: WAGNER, ~~Adolf, Fritz,~~ Volker
Residence: Germany
Citizenship: German
Post Office Address: Vischerstrasse 25, 71638 Ludwigsburg